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THE BLOOD OF *ASCIDIA NIGRA*: BLOOD CELL FREQUENCY DISTRIBUTION, MORPHOLOGY, AND THE DISTRIBUTION AND VALENCE OF VANADIUM IN LIVING BLOOD CELLS

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The vanadium concentrating ability of some tunicate species and their blood cells, particularly those cells which contain the vanadium, have long been the subject of investigation (Senozan, 1974). Theories of the function of this metal have been presented, including its possible roles in oxygen binding (Carlisle, 1968), tunicin synthesis (Endean, 1960) and antimicrobial defenses (Brown and Davies, 1971); but as yet there is no widespread agreement supporting one of these suppositions (Goodbody, 1974).

To establish the function of vanadium in the blood of ascidians, the morphological and physiological aspects of the blood should be well characterized. However, although many investigators have studied the nature of the metallic, organic and protein contents of the blood cells, procedures were employed which resulted in extensive cell lysis (Webb, 1939; Califano and Caselli, 1949; Boeri and Ehrenberg, 1954; Bielig, Bayer, Dell, Rohns, Mollinger and Rudiger, 1966), and therefore introduced significant denaturation and artifact effects. Consequently, this study was undertaken to determine blood cell frequency distribution, morphology, and the distribution and valence of vanadium in living *Ascidia nigra* blood cells. The study uses physical and chemical techniques which do not require lysis of the blood cells, *e.g.*, nuclear magnetic resonance (Carlson, 1975). Degradative techniques are used only to obtain reliable quantitative data on the total vanadium content of the blood. This study will therefore aid in understanding the function of vanadium in the blood.

Materials and Methods

Ascidia nigra specimens were collected on the coastline and surrounding reefs of Bermuda by the Bermuda Government Aquarium. They were placed in plastic bags filled with tropical sea water, packed in insulated styrofoam boxes and shipped air freight to Boston. Since the specimens were situated in unaerated water for at least eight hours, they were immediately transferred to two continuously aerated, heated 50-gallon aquaria with freshly filtered sea water and a biological filtration system upon arrival at the New England Aquarium. Water quality was monitored by routine tests and maintained with frequent water changes at the following specifications: temperature, $24.5 \pm 2.5°$ C; salinity (hand re-

fractometer, American Optical Corporation), 3.25 ± 0.05% (~0.55 M NaCl); pH (meters, models 175 and 205, Instrumentation Laboratories, Inc., Boston), 7.9 ± 0.3; dissolved oxygen (Winkler method), 6.7 ± 0.8 ppm; ammonia (Gilbert and Clay, 1973; NH_3 electrode model 95-10, meter model 407, Orion Research, Inc., Cambridge), 154 ± 84 ppb (large range due to the varying number of specimens in the tanks during a particular measurement); nitrite (Strickland and Parsons, 1955), undetected.

Cultures of *Skeletonema costatum* and *Dunaliella euchlora* grown at room temperature under fluorescent light in supplemented (half-strength) sea water, type-f (Guillard and Ryther, 1962), were added to the aquaria for food on alternate days. Unhealthy specimens, evidenced by a discoloration of the tunic, were removed and discarded as they appeared. Usually, 10–20% of the tunicates had to be removed within one week after their arrival from Bermuda, but the remainder of the specimens remained healthy until sacrificed, up to two months later.

The dissection procedure for blood withdrawal was as follows. The specimen was removed from the aquarium and gently squeezed to remove the sea water from the body. Sections of the tunic external to the heart tube were removed in thin slices until the heart was exposed. During this slicing process, spots of blood would appear on the sectioned surface of the tunic, but blood loss was insignificant, and this method permitted a quick yet careful dissection to be carried out. The heart tube was cut, and blood that welled up in the cut-away portion of the animal was collected with a plastic syringe (sterile Stylex syringe, Pharmaseal Laboratories, Glendale, California, fitted with Lancer Precision Pipette tips, Sherwood Medical Industries, St. Louis, Missouri). The openings of the plastic pipette tips were enlarged to prevent damage to the blood cells during the blood withdrawal process. If cell rupture does occur, probably resulting in the release of sulfuric acid, which is found in significant concentrations in the blood cells of other ascidians (Webb, 1939; Endean, 1955, 1960), the pH is observed to descend as low as 2–3, in agreement with past observations (Bielig, *et al.*, 1966; Senozan, 1974) and the blood cells become dark green-brown in color. Only blood samples which retained a bright yellow-green color were used for experimentation.

Plasma was separated from blood cells by centrifugation (Centrifuge model HN-S, International Equipment Co., Needham Heights, Massachusetts) for pH, salinity, and spectrophotometric analysis (Gilford Spectrophotometer Model 240, Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Using standard techniques (Bauer, 1970; Curby and Winick, 1974), whole blood was analyzed for total cell count (MK-40 hemocytometer, General Diagnostic, Bridgeport, Connecticut), cellular volume (Clay Adams microhematocrit tubes and putty, International Centrifuge Model MB), hemoglobin (iron) content (cyanomethhemoglobin, MK-40 system), and cell size (Olympus scales and microscope, Japan; Curby Bio-Detector, Grumman Corp.). Cell morphology was examined by study of blood smears (Olympus microscope) and photomicrographs (Zeiss Model RA microscope; Nikon M-35S camera) of blood slides.

Spectrophotometric analysis of whole blood was conducted in two experiments. In the first, blood was withdrawn, suspended in glycerol (Glycerol, spectro, lot # 691-A, Eastman Kodak, Rochester, New York), and analyzed in the Gilford

instrument in the presence of air. Both fresh and 4-day old blood was analyzed in this manner. In the second method, blood was withdrawn in a glove-box in a nitrogen (Suburban Welders Supply Co., Inc., Ashland, Massachusetts) environment, suspended in deaerated glycerine (Glycerine, lot #713671, Fisher Scientific Co., Fairlawn, New Jersey), and analyzed spectrophotometrically (Beckman Model 25, Fullerton, California) under argon gas (Suburban Welders Supply Co., Inc.).

Electron paramagnetic resonance studies using a Strand Labs Model 602B X-band esr spectrometer (Tuttle, Danner and Graceffa, 1972) were carried out in two experiments. The first was on blood withdrawn in a glove-box (New England Aquarium) in a nitrogen (Medical-Technical Gases, Inc., Medford, Massachusetts) environment and collected in a 50 microliter micropipet (Clay Adams, Parsippany, New Jersey) sealed with plastic clay (Seal-Ease, Clay Adams). In the second experiment, esr analysis was done on blood withdrawn in the presence of air, which had been refrigerated (6° C) for at least two weeks in a rubber-stoppered glass tube (Vacutainer, Becton, Dickinson and Co., Fairlawn, New Jersey).

Nuclear magnetic resonance studies using a modified Brucker model WH-90 nmr (Redfield and Gupta, 1971) were carried out on blood cells placed in a 5 mm glass tube (507-PP, Wilmad Glass, Buena, New Jersey). This tube was inserted into a 10 mm glass tube (513-IPP, Wilmad Glass) containing a DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) reference (Wilmad Glass, New Jersey).

Density separation of the blood cells was accomplished by centrifuging (400 *g*, 15 minutes; International Equipment Co., Centrifuge Model 80120-B) blood diluted with 2.8% saline (Abbott Laboratories, Needham, Massachusetts) which had been layered above 1.5 mol of a Lymphoprep (9.5% Sodium Metrizoate, 5.6% Ficoll; Nyegaard and Co., Oslo, Norway) medium. This is a standard medium used for the separation of human leukocytes from other type blood cells (Boyum, 1968).

Histological analysis for vanadium was accomplished through the use of osmium tetroxide (Stevens Metallurgical Corp., New York; reagent, 1800 ppm, 0.18 g OsO_4 dissolved in 100 ml 2.8% NaCl and dilute NHO_3) applied as a stain to microscope slides of whole blood. Quantitative determination of vanadium in saline solutions of cell lysates was accomplished with a standard technique (Ladd, 1974) of atomic absorption spectroscopy (Instrument Laboratories Model 153, dual beam atomic absorption spectrophotometer).

Results

Plasma characteristics

Plasma pH is essentially neutral (6.7) in *Ascidia nigra,* as it is in the plasma of *A. aspersa, A. mentula, A. fumigata,* and *A. mammillata* (Webb, 1939). Plasma salinity (3.30%) approximates that of the aquaria sea water in which the specimens resided. The ultraviolet-visible (uv-vis) absorption spectrum of a plasma sample (pH 3.1, due to rupture of some blood cells) which was yellow-green and colloidal in appearance, diluted 5:1 with distilled water (resultant pH 3.7) blanked against

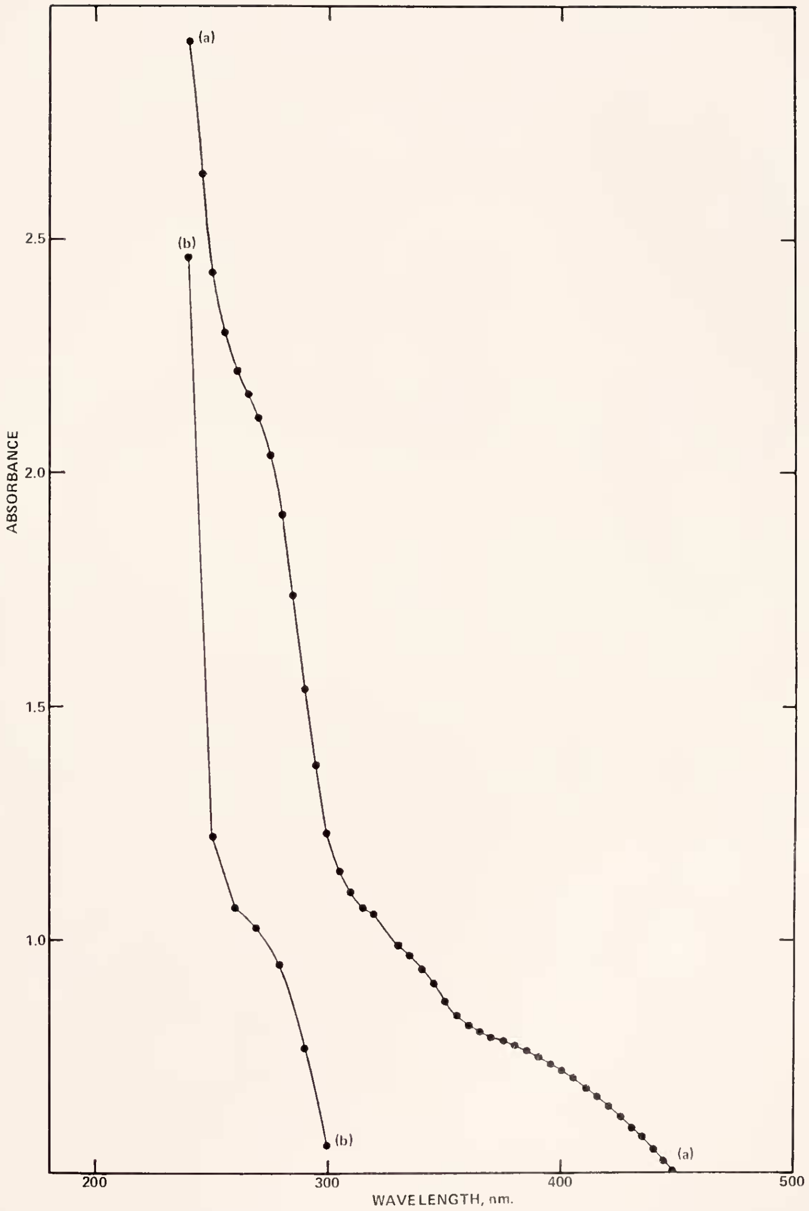

FIGURE 1. Absorption spectra of (a) plasma and (b) 1.0 N H_2SO_4, against a distilled water reference.

Table I

Differential cell counts of whole blood. Specimens 1 and 3 were the youngest of the five. Differential counts of the blood cells and volumes in the Lymphoprep layers (Tables III, IV, VI) were not obtained; therefore, Specimen 5 does not appear in those Tables.

Cell type	Large smooth	Small smooth	Green globular	Orange	Blue
Specimen 1	26%	39%	29.5%	6%	0%
Specimen 2	20.5	62	13.5	2	2
Specimen 3	40	33	14	12	0
Specimen 4	33.7	33	33	0.3	0
Specimen 5	51	38	9.5	1.5	0
Average	34.2	41.0	19.9	4.4	0.4
Absolute s.d.	11.9	12.1	10.6	4.8	0.9

distilled water, is without distinct peaks, except for a shoulder in the 260–280 nm range (Figure 1a) which is due, perhaps, to absorption by the released sulfuric acid (Figure 1b).

Blood cell characteristics, morphology, and frequency distribution

Both total blood cell count and per cent cellular volume are found to be in agreement with previously reported results for *A. nigra* (Vallee, 1967): 53,800 cells per cu mm and 1–2% cells, respectively. There is a constituent of the blood which, by the cyanmethemoglobin method, corresponds to 5.0–5.5 gram % hemoglobin; or to an iron concentration, if all the iron is in this form, of 80 μM. Cell sizes range from 2–10 microns, slightly larger than human erythrocytes, but smaller than granulocytes (Bauer, 1970). Various cell types that are observed on unstained slides correspond to earlier morphological descriptions (George, 1930, 1939; Endean, 1955, 1960), but differential counts (Vallee, 1967) of these cell types seem subject to considerable variation, due, perhaps, to the ages of the individual specimens examined, like the nonpathological differences seen in the blood of humans of different ages (Bauer, 1970).

There are four main groups of blood cells: large smooth, small smooth, green globular and orange. Large smooth cells are primarily single nucleated cells, 6–8 microns (George's vesicular signet-ring cells) and fewer "ghost" cells, 8–10 microns. Small smooth cells are primarily colorless morula types with 2–5 lobes, fewer small ghost cells, 3–4 microns, and still fewer small ghost cells with a single appendage (probably amoebocytes). Green globular cells, corresponding to George's green cells or vanadocytes are present in various sizes and with varying numbers of lobes, suggestive of different cellular developmental stages. Orange cells are those with a very bright orange pigment.

Cells that are infrequently found are blue cells, brown cells, and cells with basophilic-like stippling, 8–10 microns in diameter. As to the relative frequency of occurrence of the four main groups of cells (Table I), large smooth, small smooth, and green globular cells are significantly more abundant (34.3%, 41.0%, and 19.9%, respectively) than the orange cells, which are the least (4.4%) abun-

dant. Younger specimens seem to have higher percentages of orange cells (6% and 12%), which is significantly higher than figures previously reported (Vallee, 1967).

The blood of a young specimen was observed to develop two layers of cells on standing in a glass tube for five to ten minutes: an orange top layer consisting of large smooth, small, and orange cells, and a yellow-green "pellet" primarily composed of green globular cells (Table II). Total cell counts for each of these two layers were tabulated on the Curby Bio-Dectector: the top layer having 14,508 cells per cu mm; the pellet, 64,341 cells per cu mm. If blood samples, therefore, are not adequately agitated to produce homogeneous mixtures, this settling phenomenon (which is observable in human blood only over a period of several hours) may account for two findings: the variation in differential cell counts (Table I) of whole blood, and the wide range of total cell counts reported for *A. nigra* [31,600–79,390 cells per cu mm (Vallee, 1967)] and for *Pyura stolonifera* [18,250–68,200 cells per cu mm (Endean, 1955)]. Another possible source of this variation is the possibility that it stems from variations in the rate of influx to the blood of undifferentiated or partially differentiated blood cells and/or variations in the rate of efflux from the blood of fully differentiated or senescent cells.

Although the results of histological staining will provide more conclusive evidence, the presence of various transitory stages of cells in unstained blood (as well as the results of the Lymphoprep density centrifugation, discussed below) suggest a maturational sequence which would be in fair agreement with past hypotheses (Endean, 1960; Kalk, 1963): the large smooth cell becomes a colorless morula-shaped cell, which changes into the green vanadocyte.

Ultraviolet-visible absorption of blood

The uv-vis absorption spectrum of intact blood cells suspended in glycerol (glycerine), blanked against the same, demonstrates the effect of exposure to air,

TABLE II

Differential cell counts of blood allowed to stand five to ten minutes.

Cell type	Large smooth	Small smooth	Green globular	Orange	Blue
Top layer	26%	56.5%	5.5%	12.5%	0%
"Pellet"	26	14	52	5.5	2
Number of cells per cu mm, and as % of 53,800					
Top layer	13,988	30,397	2,959	6,725	0
"Pellet"	13,988	7,532	27,976	2,959	1,076
Weighted average	13,988	18,965	15,468	4,842	538
Per cent	26.0	35.3	28.8	9.0	1.0
Per cent ranges (Table I)	22.3–46.1	28.9–53.1	9.3–30.5	0–9.2	0–1.3

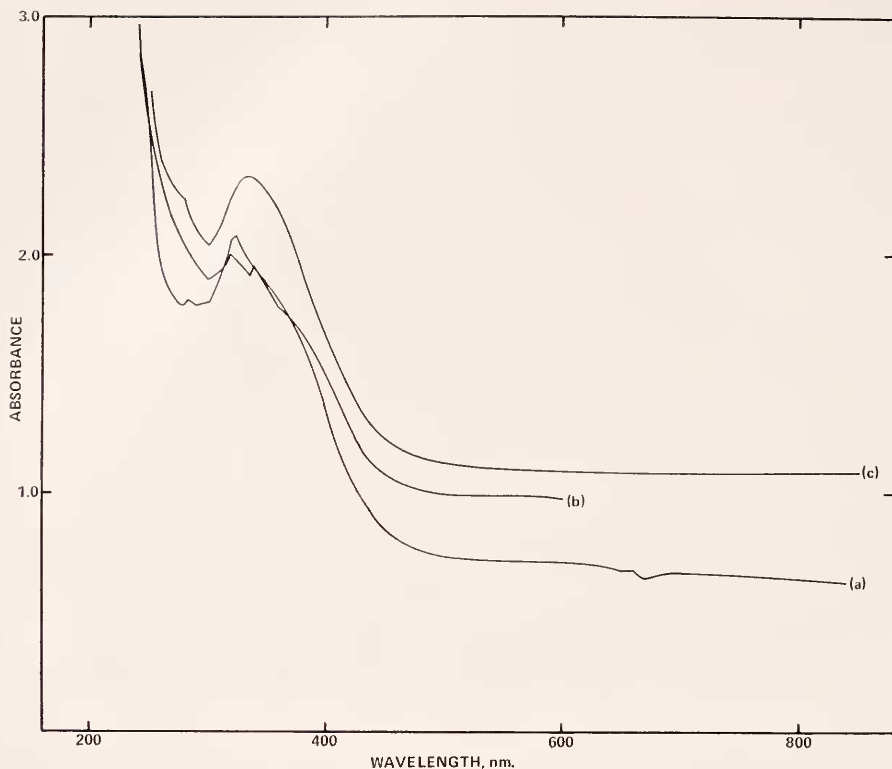

FIGURE 2. Absorption spectra of blood cells. Curves (a) and (c) were taken immediately after dissection; (a) for aerated and (c) for unaerated blood. Spectrum (b) is for four-day old refrigerated blood. In each case the cells were suspended in glycerol and blanked against the same.

as well as the effect of cell lysis. Blood which is isolated from atmospheric oxygen (Fig. 2c) shows an absorbance maximum at 335 nm and a shoulder absorbance in the vicinity of 280 nm. Blood which is allowed to come into contact with air (Fig. 2a) shows a shift of its absorption maximum to 325 nm. Four day old blood, exposed to air and refrigerated (Fig. 2b) has two absorbance maxima at 325 and 340 nm. Additionally, the positions of all of the above intact blood cell absorption maxima differ significantly from maxima reported for different forms of lysed blood cell solutions at 275, 280, 300, 420, and 610 nm (Bielig, Bayer, Califano and Wirth, 1954).

Magnetic resonance analysis of blood

Electron paramagnetic resonance (epr). The eight-line spectrum (Carrington and McLachlan, 1967) of VO^{2+} ion in acidic medium was clearly visible in a 0.001 M vanadyl sulfate solution at 20° C. The epr line spectrum of V^{3+} has not been observed in solution as it is too broad (Carrington and McLachlan, 1967).

Blood extracted in a nitrogen environment shows no epr signal. Air-oxidized, two week old blood yielded a VO^{2+} signal roughly comparable to that of the millimolar reference. Vanadium present in the blood cells is therefore in the +3 valence state. The relatively low concentration of vanadium(IV) in the aged sample is probably due to the fact that oxidation proceeds to the +5 state. The presence of V(III) in *A. nigra* is in agreement with the conclusion (Swinehart *et al.,* 1974) that in vanadium-concentrating species (order: Enterogona) the +3 valence state is more common among species of sub-order Phlebobranchiata, of which the Ascidiidae are members (Millar, 1966).

Nuclear magnetic resonance (*nmr*). The proton nmr spectrum of a centrifuged blood sample was taken at 22° C. The spectrum consists of two resonance lines: a very strong, essentially Lorentzian signal approximately 5 ppm downfield from a DDS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) reference; and a weaker, apparently Gaussian signal with a peak about 20 ppm from the DDS reference line. These features are consistent with an earlier report on the ^1H nmr spectrum of living blood cells of *A. ceratodes* collected on the Pacific coast (Carlson, 1975). The previous assignment of the strong signal to plasma water and the weaker, 20 ppm resonance to vanadophore solvent water broadened by paramagnetic V(III) is also supported by the *A. nigra* spectra. However, the width of the line we have measured appears to be considerably narrower; the full width at half height is approximately 600 Hz. If the nuclear relaxation process is controlled by the rate of chemical exchange, as it is for dilute aqueous V^{3+} (Chmelnick and Fiat, 1972), the line should narrow with decreasing temperature, and the *A. nigra* nmr spectrum at 22° C. would then be consistent with that of *A. ceratodes* at 33° C.

Taken together, the epr and nmr studies indicate the existence of a tripositive, partially complexed vanadium species in the blood cells. The inference of further conclusions on the structure of the vanadium complex from the resonance data does not seem to be justified at this time.

Lymphoprep density centrifugation of blood

Density centrifugation of saline-diluted blood on Lymphoprep medium results in the formation of five layerings (Fig. 3a) of fairly uniform volumes (Table III)

Table III

Results of lymphoprep density centrifugation (volumes in ml).

Specimen	Volume whole blood	Volume saline added	Total volume	Cell band volumes and percentages of total volume									
				1	%	2	%	3	%	4	%	5	%
1	0.70	4.30	6.5	4.50	69.2	0.25	3.8	0.60	9.2	0.80	12.3	0.10	1.5
3	0.50	3.00	5.0	1.83	36.6	0.67	13.4	0.67	13.4	0.11	2.2	0.07	1.4
5	0.30	1.80	3.6	2.48	68.6	0.73	20.3	0.51	14.2	0.22	6.1	0.05	1.4
Average					58.2		12.5		12.3		6.9		1.4
Absolute s.d.					18.7		8.3		2.7		5.1		0.1
2	1.30	3.70	6.5	4.70	72.3	0.35	5.4	1.15	17.7	0.13	2.0	—	—

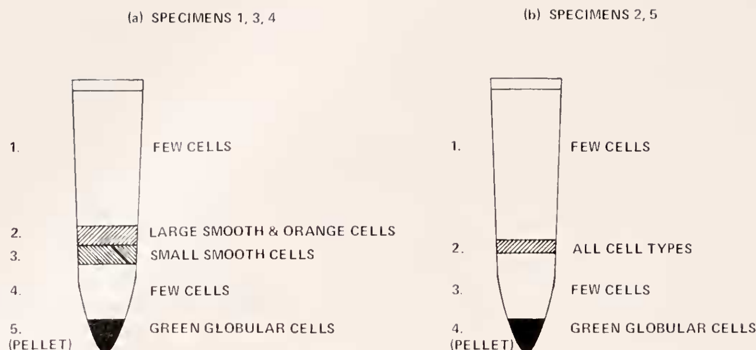

FIGURE 3. Results of lymphoprep density centrifugation.

or four layerings (Fig. 3b). Layers 2 and 3 (which consist of lymphocytes when human blood is centrifuged in Lymphoprep) are composed of large smooth and orange cells, and small smooth cells, respectively. Layer 5, the pellet (which would consist of erythrocytes of human blood), is primarily composed of green globular cells (Table IV). Note that in this and succeeding tables layer 2 of specimen 4 will be considered with layers 2 and 3 of specimens 1, 3, and 4, since the former did not differentiate the lower density cell layer. The relative frequencies of the blood cells in a particular layer and the layer preferences of the cells (Table IV), indicative of the density differences, correspond to the suggested maturational sequence of large smooth to small smooth to green globular.

Vanadium content of blood

Application of the OsO_4 reagent (or a 50% dilution of the reagent with 2.8% saline) produces blackening of the interiors of the green globular cells, with no observed discoloration of any of the other cell types or plasma in slides of whole blood. It seems probable, therefore, that trivalent vanadium in these green globular cells (green cells, vanadocytes) is the cause of the chemical reduction of OsO_4 (George, 1939; Webb, 1939).

Overall vanadium concentrations of whole blood samples determined by atomic absorption spectroscopy (Table V) range from 2.54–23.44 mM, with an average of 10.73 mM. Vanadium concentration of the Lymphoprep density centrifugation layers (Table VI) corresponds directly to the green globular vanadocyte layer preference (Table IV) of Layer 5 > Layer 2 > Layer 3; and some of these cells could be reasonably expected to be found in layers 4 and 1.

DISCUSSION

Within a short time after cell rupture, the lysate has a different hue from the intact cells. The specific color obtained is a function of the age and degree of oxidation of the solution. For example, the yellow-brown lysate solution previously reported (Boeri and Ehrenberg, 1954) is probably a hydrolytic dimer of

TABLE IV

Differential cell counts of lymphoprep density centrifugation layers. Note that the relative frequencies of the cell types in the layers are: layer 2, large smooth > orange > green globular > small smooth; layer 3, small smooth > green globular > orange > large smooth; layer 5, green globular > large smooth > small smooth > orange. The layer preferences of the cells are: large smooth, 2 > 5 > 3; small smooth, 3 > 5 > 2; green globular, 5 > 2 > 3; orange, 2 > 3 > 5.

Cell type	Large smooth	Small smooth	Green globular	Orange
Specimen 1, layer 2	47%	9.5%	4%	40.5%
Specimen 2, layer 2	14	15	37.5	33.5
Specimen 3, layer 2	16	6	33.5	44.5
Specimen 4, layer 2	71.5	22.5	5	1
Average	37.1	13.3	20.0	29.9
Absolute s.d.	27.4	7.2	18.0	19.8
Specimen 1, layer 3	7.5	63.5	5	24
Specimen 2, layer 2	14	15	37.5	33.5
Specimen 3, layer 3	13.5	57	23.5	6
Specimen 4, layer 3	7	88.5	3.5	1
Average	10.5	56.0	17.4	16.1
Absolute s.d.	3.8	30.5	16.2	15.2
Specimen 1, layer 5	22.5	8	68	1.5
Specimen 2, layer 4	20.5	32	46	2
Specimen 3, layer 5	43.5	5	47.5	4
Specimen 4, layer 5	25.5	22.5	50.5	1.5
Average	28.0	16.9	53.0	2.3
Absolute s.d.	10.5	12.7	10.2	1.2

vanadium(III) (Newton and Baker, 1964). This and other examples of dissociation between metal and complexing ligand, oxidative reactions of metal and ligand, and degradation of protein show that the correct nature of the vanadium

TABLE V

Vanadium content of whole blood.

Specimen	[V], millimolar	ppm, or milligrams/liter
1	23.44	1195
2	11.82	603
3	12.15	620
4	3.72	190
5	2.54	130
Average	10.73	548
Absolute s.d.	8.38	427

TABLE VI

Vanadium content of Lymphoprep density centrifugation layers. Per cent cells assumes a cell volume of 320μ³ (Webb, 1939). Average (−2) is the average excluding Specimen 2 data.

Specimen	Layer [V], mM	Layer volume ml	Per cent total volume	Vanadium concentration micromoles	Per cent total V	Layer cell count per cu mm	Per cent cells
Specimen 1, layer 1	0.0	4.50	69.2	0.0	0.0	654	0.021
Specimen 2, layer 1	0.0	4.70	72.3	0.0	0.0	242	0.008
Specimen 3, layer 1	0.54	1.83	36.6	0.99	13.81	175	0.006
Specimen 4, layer 1	0.41	2.48	68.8	1.02	40.32	109	0.003
Average (−2)	0.24	3.38	61.7	0.50	13.53	295	0.010
Absolute s.d.	0.28	1.44	16.8	0.58	19.01	245	0.008
Average	0.32	2.94	58.2	0.67	18.04	313	0.010
Absolute s.d.	0.28	1.39	18.7	0.58	20.49	297	0.009
Specimen 1, layer 2	12.83	0.25	3.8	3.21	23.67	12278	0.39
Specimen 2, layer 2	7.62	0.35	5.4	2.67	13.50	6646	0.21
Specimen 3, layer 2	2.72	0.67	13.4	1.82	25.38	2930	0.09
Specimen 4, layer 2	1.19	0.73	20.3	0.87	34.39	2816	0.90
Average (−2)	6.09	0.50	10.7	2.14	24.24	6168	0.20
Absolute s.d.	5.26	0.24	7.6	1.02	8.56	4445	0.14
Average	5.58	0.55	12.5	1.97	27.81	6008	0.19
Absolute s.d.	6.33	0.26	8.3	1.18	5.76	5430	0.17
Specimen 1, layer 3	8.77	0.60	9.2	5.26	38.79	9276	0.30
Specimen 2, layer 2	7.62	0.35	5.4	2.67	13.50	6646	0.21
Specimen 3, layer 3	4.62	0.67	13.4	3.10	43.24	4015	0.13
Specimen 4, layer 3	0.60	0.51	14.2	0.31	12.25	2514	0.08
Average (−2)	5.40	0.53	10.6	2.83	26.95	5613	0.18
Absolute s.d.	3.65	0.14	4.1	2.03	16.36	2980	0.10
Average (−2)	4.66	0.59	12.3	2.89	31.43	5268	0.17
Absolute s.d.	4.09	0.08	2.7	2.48	16.76	3551	0.12
Specimen 1, layer 4	4.51	0.80	12.3	3.61	26.62	1309	0.04
Specimen 2, layer 3	9.52	1.15	17.7	10.95	55.39	1038	0.03
Specimen 3, layer 4	3.20	0.11	2.2	0.35	4.88	2986	0.10
Specimen 4, layer 4	0.64	0.22	6.1	0.14	5.53	1689	0.05
Average	4.47	0.57	9.6	3.76	23.11	1756	0.06
Absolute s.d.	3.73	0.49	6.8	5.05	23.77	863	0.03
Average (−2)	2.78	0.38	6.9	1.37	12.34	1995	0.06
Absolute s.d.	1.97	0.37	5.1	1.95	12.37	879	0.03

TABLE VI—(*Continued*)

Specimen	Layer [V], mM	Layer volume ml	Per cent total volume	Vanadium concentration micromoles	Per cent total V	Layer cell count per cu mm	Per cent cells
Specimen 1, layer 5	14.83	0.10	1.5	1.48	10.91	197838	6.36
Specimen 2, layer 4	47.34	0.13	2.0	6.15	31.11	160900	5.15
Specimen 3, layer 5	12.94	0.07	1.4	0.91	12.69	101586	3.25
Specimen 4, layer 5	3.86	0.05	1.4	0.19	7.51	82273	2.63
Average	19.74	0.09	1.6	2.18	15.56	135874	4.35
Absolute s.d.	19.01	0.04	0.3	2.70	10.59	53625	1.72
Average (−2)	10.54	0.07	1.4	0.86	10.37	127532	4.08
Absolute s.d.	5.86	0.03	0.1	0.65	2.63	62417	2.00

binding and of the "chromogen" in the intact cell is not readily inferred from lysate data. Earlier (Webb, 1939) it had been suggested that the "chromogen" in vanadocytes is a bile pigment pyrrole, perhaps biliverdin (Lemberg and Legge, 1949). It is very likely, however, that the positive test for pyrroles resulted from the presence of hemoglobin in the lysate. Is it then likely that the spectrum of the intact vanadocyte is primarily due to a vanadium complex?

The electronic configuration of V(III) is [A] ($3d^2$), and attempts to provide spectral data on a $3d^2$ system in octahedral symmetry have therefore centered on V^{3+} ion in different compounds (Ballhausen, 1962). In aqueous solution $V(H_2O)_6^{3+}$ is blue, with maxima at 575 and 397 nm in the optical region. The bands have been successfully assigned to energy-level transitions based on ligand field theory. Shifts of the bands occur as a result of changes in ligand field strength; relative positions may also change when the symmetry of the field is changed. These effects can be accounted for satisfactorily in terms of the ligand field model (Cotton and Wilkinson, 1972; Ballhausen, 1962). None of the reported spectra resemble the whole blood spectrum shown in Figure 2. Consequently, the vanadium(III) complex is probably not involved to a significant extent in the optical absorption spectrum of *A. nigra's* yellow-green blood cells. The possibility that the absorption spectrum of the blood cells is due mainly to a wholly organic compound should then be considered; for example, comparison with chlorophyll and other hematin compounds may be made.

However, none of these compounds possess absorption spectra resembling that of the intact blood cells. Chlorophyll and the bile pigment biliverdin have optical spectra characterized by blue and red bands. The vanadocyte spectrum lacks the red band, and the peak at 320–325 nm lies too deeply in the uv to be a blue band. Absorption spectra of hemoglobin compounds (Lemberg and Legge, 1949) all show strong peaks at 400–450 nm which do not correlate with Figure 2. Thus, hematin-like complexes are probably neither responsible for the color of the blood cells, nor present to an extensive degree.

The closest meaningful spectral correlation between ascidian blood cells and a well defined compound that we have been able to find is with pteridines (Karrer,

Manunta and Schwyzer, 1948; Gaill and Momzikoff, 1975). The relevant optical properties shown by pteridines are two to four uv bands (the spectra are strongly pH dependent due to tautomerism), and a maximum above 300 nm, but none above 400 nm (Blakeley, 1969). Xanthopterin, a pteridine obtained from *Microcosmus polymorphus*, has maxima at 281 and 373 nm, and exhibits fluorescence. The blood cell spectra (Fig. 2a and 2c) show bands in the uv and below 400 nm; no visible fluorescence of the whole blood cells is observed upon uv irradiation, however.

It is obvious that whole blood cells are complex mixtures for which a simple spectral assignment would not be anticipated (Swinehart, Biggs, Halko, and Schroeder, 1974). It is equally clear, however, that characteristics of the blood cell lysate, unless obtained and studied under conditions that are essentially intracellular, are misleading indicators of the chemical constitution of the blood cell. An organic compound dominates the absorption spectrum. This compound may be a pteridine, in which case the lack of fluorescence may be due to conditions within the cell.

This conclusion has interesting ramifications, for though *M. polymorphus* and *A. nigra* are in the class Ascidiacea, members of the family Ascidiidae possess vanadocytes, whereas members of the family Pyuridae (of which *M. polymorphus* is one) have ferrocytes (Endean, 1955; Smith, 1970) and are usually low in vanadium content (Swinehart *et al.*, 1974). The similarity in morphology, organic composition and structure of the blood cells, together with the similarity in appearance and ecology of the two genera suggest two successful evolutionary lines, in one of which (Ascidiidae) vanadium(III) or (IV) has the same function that iron(II) has in the other (Pyuridae) line.

The relative vanadium concentration of the layers, $5 > 2 > 3 > 4 > 1$, corresponds to the relative total cell count of the layers (Table VI). It would not be unusual for vanadium to be present in cells other than the green globular cells, and be of an oxidation state (pentavalent) that would not reduce OsO_4. This would imply that the relative vanadium concentrations of the layers are indicative of the overall cell counts, not the green globular cell frequencies. Data (Table VI) which correct for concentration effects and reflect the vanadium *contribution* by a particular layer toward the total vanadium in the blood (% Total V) show that each layer makes a significant contribution. This calculation suggests that vanadium is present in additional cell types.

It should be noted, however, that the cells in all of the Lymphoprep density centrifugation layers make up a very small part of a particular layer's total volume. It is possible that vanadium, in free or bound form, escapes into the soluble portions of the layers as a result of cell rupturing. Cell rupturing, if significant, would tend to erase the differences in vanadium concentration between layers, and produce a leveling effect.

Tunicates concentrate vanadium in the pentavalent state from sea water (Ladd, 1974; McLeod, Ladd, Kustin, and Toppen, 1975) and must obviously reduce the metal to either a trivalent or tetravalent oxidation state, since V(III) or V(IV) is present, depending on the tunicate order (Swinehart *et al.*, 1974). It is conceivable that less mature blood cells in a developmental scheme, such as the large

smooth and small smooth cells, would possess pentavalent vanadium, which is suggested by the atomic absorption metal analyses of the Lymphoprep density centrifugation layers. During maturation, in the case of *Ascidia nigra*, the pentavalent vanadium would be reduced to the trivalent form, the presence of which is indicated by the lack of a vanadium signal in the epr spectrum and the OsO_4 staining. This hypothesis is similar to the actual mechanism of iron incorporation which occurs in human erythrocytes (Bloom and Fawcett, 1968; Harper, 1973). Coincident with this chemical reduction of vanadium, protein synthesis would be occurring in the cells, resulting in an increase in the density of the cells, which is reflected in the results of the Lymphoprep density centrifugation.

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Summary

1. The blood plasma of *Ascidia nigra* has been characterized with regard to pH, salinity, and ultraviolet-visible absorption. Whole blood of *A. nigra* has been characterized with regard to cell count, cellular volume, hemoglobin (iron) content, vanadium content. Blood cell types have been examined, categorized, and differentially counted.

2. Epr and nmr studies prove that trivalent vanadium is present. Staining of whole blood slides with OsO_4 reagent identify the vanadium-carrying cells as being mainly the green globular blood cells. The observed uv-vis absorption spectrum does not correlate well with known vanadium(III) complex spectra.

3. Density separation of blood cells has been achieved; this result coupled with various cell morphologies suggests that different cells may represent different maturational stages in a developmental process.

4. Vanadium analyses of layerings of cells of different densities suggest that vanadium may be present in more than one type of blood cell, where, when not present as vanadium(III), it would be present in the diamagnetic, pentavalent oxidation state.

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